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<b>(21) International Application Number:</b> PCT/US95/07855 <b>(22) International Filing Date:</b> 23 June 1995 (23.06.95) <b>(30) Priority Data:</b> 08/271,667 7 July 1994 (07.07.94) US <b>(60) Parent Application or Grant</b> (63) Related by Continuation US 08/271,667 (CIP) Filed on 7 July 1994 (07.07.94) <b>(71) Applicants (for all designated States except US):</b> UNIVER- SITY OF MARYLAND AT BALTIMORE [US/US]; 511 West Lombard Street, Baltimore, MD 21201-1691 (US). PHARMACIA S.P.A. [IT/IT]; Via Robert Koch, 1.2., I- 20152 Milan (IT). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> BENATTI, Luca [IT/IT]; Via Tagliabue, 11, I-20093 Cologno Monzese (IT). BRE- TON, Jerome [FR/IT]; Via A. Sforze, 61, I-20136 Milan (IT). SPECIALE, Carmela [IT/IT]; Via Giovanni XXIII, 21, I-20124 Nerviano (IT). OKUNO, Etsuo [JP/JP]; 1675- 2, Kinomoto, Wakayama 640 (JP). SCHWARCZ, Robert		<b>[IT/IT];</b> 6936 Ten Timbers Lane, Baltimore, MD 21209 (US). MOSCA, Monica [IT/IT]; Via dei Gracchi, 26, I- 20100 Milano (IT). <b>(74) Agents:</b> BIGGART, Waddell, A. et al.; Sughrue, Mion, Zinn, MacPeak & Seas, Suite 800, 2100 Pennsylvania Avenue, N.W., Washington, DC 20037-3202 (US). <b>(81) Designated States:</b> CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> RECOMBINANT KAT ENZYME AND PROCESS FOR ITS PREPARATION		
<b>(57) Abstract</b> <p>Disclosed are isolated DNAs encoding a kynurenine aminotransferase selected from the group consisting of: (a) isolated DNA sequences which encode rat KAT; (b) an isolated DNA sequence which hybridizes to isolated DNA sequences of (a) above and which encodes a mammalian KAT enzyme; and (c) an isolated DNA sequence differing from the isolated DNA sequences of (a) and (b) above in codon sequence due to the degeneracy of the genetic code, and which encodes a KAT enzyme. Vectors and host cells containing the same, oligonucleotide probes for identifying kynurenine aminotransferase, and isolated and purified kynurenine aminotransferase are also disclosed.</p>		

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## RECOMBINANT KAT ENZYME AND PROCESS FOR ITS PREPARATION

### FIELD OF THE INVENTION

The present invention relates to DNA sequences that code for kynurenine aminotransferase.

### BACKGROUND OF THE INVENTION

5           The enzyme kynurenine aminotransferase (known in the art as KAT) catalyzes the biosynthesis of kynurenic acid (KYNA) from kynurenine (KYN) and is singularly responsible for the regulation of extracellular KYNA concentrations in the brain  
10           (J. Neurochem., 57:533-540 (1991)).

          KYNA is an effective excitatory amino acid (EAA) receptor antagonist with a particularly high affinity to the glycine modulatory site of the N-methyl-D-aspartate (NMDA) receptor complex  
15           (J. Neurochem., 52:1319-1328 (1989)). As a naturally occurring brain metabolite (J. Neurochem., 51:177-180 (1988); and Brain Res., 454:164-169 (1988)), KYNA probably serves as a negative endogenous modulator of cerebral glutamatergic function (Ann. N.Y. Acad. Sci.,  
20           648:140-153 (1992)).

          EAA receptors and in particular NMDA receptors are known to play a central role in the function of the mammalian brain (Watkins et al, In: The NMDA Receptor, page 242, (1989), Eds., Oxford University  
25           Press, Oxford). For example, NMDA receptor activation is essential for cognitive processes, such as, for example, learning and memory (Watkins et al, In: The NMDA Receptor, Eds., pages 137-151, (1989), Oxford University press, Oxford) and for brain development  
30           (Trends Pharmacol. Sci., 11:290-296 (1990)).

          It follows that a reduction in NMDA receptor function will have detrimental consequences for brain

physiology and, consequently, for the entire organism. For example, the decline in the number of NMDA receptors which occurs in the aged brain (Synapse, 6:343-388 (1990)) is likely associated with age-related disorders of cognitive functions.

In the brain, KYNA concentrations and the activity of KYNA's biosynthetic enzyme KAT show a remarkable increase with age (Brain Res., 558:1-5, (1992); and Neurosci. Lett., 94:145-150 (1988)). KAT inhibitors, by providing an increase of the glutamatergic tone at the NMDA receptor, could therefore be particularly useful in situations where NMDA receptor function is insufficient and/or KAT activity and KYNA levels are abnormally enhanced. Hence they could be particularly useful in the treatment of the pathological consequences associated with the aging processes in the brain which are, for example, cognitive disorders including, e.g., attentional and memory deficits and vigilance impairments in the elderly.

KAT inhibitors may also be useful in the treatment of perinatal brain disorders which may be related to irregularities in the characteristic region specific pattern of postnatal KAT development (Baran et al, Dev. Brain Res., 74:283-286 (1993)).

In subcellular fractionation studies KAT activity was recovered either in the cytosol and in mitochondria (J. Neurochem., *supra*).

Most nuclear-encoded precursors of mitochondrial proteins contain amino-terminal presequences (Pfanner et al, In: Current Topics in Bioenergetics, 15:177-219 (1987); Lee Ed., New York Academic Press; and Nicholson et al, In: Protein Transfer and Organelle Biogenesis, Das and Robins Eds., New York Academic Press (1988)). These presequences are required for the precursor to enter the mitochondrial matrix, where they are proteolytically removed (Hurt et al, FEBS Lett., 178:306 (1984);

- 3 -

Horwich et al, EMBO J., 4:1129 (1985). This cleavage is not essential for completing import but is necessary for further assembly of the newly imported polypeptides into functional complexes (Zwizinski et al, J. Biol. Chem., 258:13340 (1983); Lewin et al, J. Biol. Chem., 258:6750 (1983); Ou et al, J. Biochem., 100:1287 (1986)). Precursor targeting sequences differ considerably in their structures. One of the few common themes is the high content of positively charged amino acids and of hydroxylated amino acids. Presequences may form an amphipathic structure in the form of either  $\alpha$ -helices or  $\beta$ -sheets (von Heijne et al, EMBO J., 5:1335 (1986); Roise et al, EMBO J., 5:1327 (1986); and Vassarotti et al, EMBO J., 6:705 (1987)). Despite the large variability of the sequences of mitochondrial leader peptides, relatively minor alterations of the presequence can prevent cleavage by the processing peptidase (Hurt et al, J. Biol. Chem., 262:1420 (1987)). This suggests that distinct, but up to now undefined, structural elements are required for cleavage. Similarly, the cleavage sites show wide variation among different precursors of a single organism and among precursors of different organisms.

Interestingly, using the protein algorithm described by Gavel et al (Protein Engineering, 4:33-37 (1990)), a potential mitochondrial transit peptide is predicted either in position 1 to 24 of the deduced protein of cDNA-2 and in position 1 to 44 of the deduced protein of cDNA-3 disclosed in the present invention (see Figures 3-4 and Example 3).

Recently Perry et al (Mol. Pharm., 43:660-665 (1993)) reported the cloning of a cDNA coding for rat kidney cytosolic cysteine conjugate  $\beta$ -lyase. When the cDNA was inserted into the expression vector pVS1000 and

- 4 -

transfected into COS-1 tissue culture cells, a 7-10 fold increase in cytosolic  $\beta$ -lyase and glutamine transaminase K activities were detected.

The deduced amino acid sequence of rat  $\beta$ -lyase is identical to the deduced amino acid sequence of cDNA-1 (rat KAT) except for two residues (see Figure 2). Moreover the existence of cDNA-2 and cDNA-3 was not reported by Perry et al (Mol. Pharm., *supra*).

Even more recently Perry et al (FEBS Lett., 360:277-280 (1995)) reported the cloning of a cDNA for human kidney cysteine conjugate beta-lyase whose sequence is identical to the sequence of the human KAT described in the present patent application.

Whereas the identity with cysteine conjugate  $\beta$ -lyase and glutamine transaminase K is well documented (Abraham et al, Analytical Biochem., 197:421-427 (1991)), there are no reports indicating identity of kynurenine transaminase neither with  $\beta$ -lyase nor with glutamine transaminase K.

#### SUMMARY OF THE INVENTION

We now report the cloning of mammalian kynurenine aminotransferases.

A first aspect of the present invention are isolated DNA sequences encoding a KAT enzyme selected from the group consisting of: (a) isolated DNA sequences which encode rat KAT; (b) an isolated DNA sequence which hybridizes to isolated DNA sequences of (a) above and which encodes a mammalian KAT enzyme; and (c) an isolated DNA sequence differing from the isolated DNA sequences of (a) and (b) above in codon sequence due to the degeneracy of the genetic code, and which encodes a KAT enzyme.

- 5 -

A second aspect of the present invention are vectors comprising a cloned DNA sequence as given above.

A third aspect of the present invention are host cells transformed with a vector as given above.

A fourth aspect of the present invention is an oligonucleotide probe capable of selectively hybridizing to a DNA comprising a portion of a gene coding for a KAT enzyme.

A fifth aspect of the present invention is isolated and purified KAT enzyme which is coded for by a DNA sequence selected from the group consisting of: (a) isolated DNA sequences which encode rat KAT; (b) isolated DNA sequence which hybridizes to isolated DNA sequence of (a) above and which encodes a mammalian KAT enzyme; and (c) an isolated DNA sequence differing from the isolated DNA sequences of (a) and (b) above in codon sequence due to the degeneracy of the genetic code, and which encodes a KAT enzyme.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 Partial amino acid sequence of rat KAT: N-terminus of mature KAT (SEQ ID NO:14), a CNBr fragment (SEQ ID NO:15), tryptic fragment 112 of KAT (SEQ ID NO: 16) and tryptic fragment 130 of KAT (SEQ ID NO:17).

Figures 2A-2C nucleotide sequence and deduced amino acid sequence of rat KAT (cDNA-1) (SEQ ID NO:18). The putative pyridoxal phosphate binding site, Ser-Ala-Gly-Lys-Ser-Phe, is underlined. Triplets differing from rat  $\beta$ -lyase cDNA (Perry et al, supra) are boxed.

Figures 3A-3D nucleotide sequence and deduced amino acid sequences of rat KAT (cDNA-2) (SEQ ID

- 5/1 -

NO:19). Two proteins can be synthesized: one starting  
from

- 6 -

nucleotide 619 and including a putative mitochondrial targeting peptide, the other beginning at the same ATG starting codon as in the case of cDNA-1. The putative pyridoxal phosphate binding site, Ser-Ala-Gly-Lys-Ser-Phe, is underlined. Triplets differing from rat  $\beta$ -lyase cDNA (Perry et al, supra) are boxed.

Figures 4A-4D nucleotide sequence and deduced amino acid sequences of rat KAT (cDNA-3) (SEQ ID NO:5). The sequence of cDNA-3 is identical to that of cDNA-1 except for an insertion of 208 base pairs in the 5'-untranslated region. The insertion creates an additional stretch of 34 amino acids in frame with the cDNA-1 deduced protein sequence. The insertion of these 208 base pairs occurs between nucleotide 237 and 238 of the cDNA-1 sequence.

Figures 5A and 5B cytosolic enzyme activities in transfected COS-1 cells: 5A, glutamine transaminase K activity; 5B, kynurenine transaminase activity. Sense: pSVL-KAT transfected COS-1 cells were cDNA-1 is in the sense orientation. Antisense: pSVL-KAT transfected COS-1 cells were cDNA-1 is in reverse orientation. Each value is the mean of three separate experiments.

Figure 6 Partial amino acid sequence of human KAT I: tryptic fragments F11 (SEQ ID NO:2); F13 (SEQ ID NO:3); and F14 (SEQ ID NO:4) of the human KAT I.

Figures 7A-7C nucleotide sequence and deduced amino acid sequence of human KAT I (SEQ ID NO:1).

#### DETAILED DESCRIPTION OF THE INVENTION

Amino acid sequences disclosed herein are presented in the amino to carboxy direction, from left to right. The amino and carboxy groups are not presented in the sequence. Nucleotide sequences are

- 7 -

presented herein by single strand only, in the 5' to 3' direction, from left to right. Nucleotides and amino acids are represented herein in the manner recommended by the IUPAC-IUB Biochemical Nomenclature Commission, or (for amino acids) by three letter code.

The kynurenine aminotransferase enzyme of the present invention includes proteins homologous to, and having essentially the same biological properties as, the protein coded for by the nucleotide sequences herein disclosed. This definition is intended to encompass natural allelic variants of KAT sequence.

Cloned genes of the present invention may code for KAT of any species of origin, but preferably code for enzymes of mammalian origin. Thus, DNA sequences which hybridize to the sequences given in Figures 2A-2C (SEQ ID NO:18), 3A-3D (SEQ ID NO:19), 4A-4D (SEQ ID NO:5) and 7A-7C (SEQ ID NO:1) and which code for expression of KAT are also an aspect of this invention. Conditions which will permit other DNA sequences which code for expression of KAT to hybridize to the sequences given in Figures 2A-2C (SEQ ID NO:18), 3A-3D (SEQ ID NO:19), 4A-4D (SEQ ID NO:5) and 7A-7C (SEQ ID NO:1) can be determined in a routine manner. Further, DNA sequences which code for polypeptides coded for by the sequences given in Figures 2A-2C (SEQ ID NO:18), 3A-3D (SEQ ID NO:19), 4A-4D (SEQ ID NO:5) and 7A-7C (SEQ ID NO:1) or sequences which hybridize thereto and code for a KAT enzyme, but which differ in codon sequence from these due to degenerancy of the genetic code, are also an aspect of this invention. The degenerancy of the genetic code, which allows different nucleic acid sequences to code for the same protein or peptide, is well known in the literature. See, e.g., Toole et al, U.S. Patent 4,757,006 at column 2, Table 1.

- 8 -

DNA which encodes the KAT enzyme may be obtained by a variety of means well known to the expert in the art and disclosed by, for example, Maniatis et al, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Press, Cold Spring Harbor, NY (1989).

For example, DNA which encodes the KAT enzyme may be obtained by screening of mRNA or genomic DNA with oligonucleotide probes generated from the KAT enzyme gene sequence information provided herein. Probes may be labeled with a detectable group such as a fluorescent group, a radioactive atom or a chemiluminescent group in accordance with known procedures and used in conventional hybridization assays, as described by, for example, Maniatis et al, *supra*.

KAT gene sequences may alternatively be recovered by use of the polymerase chain reaction (PCR) procedure, with the PCR oligonucleotide primers described herein or with oligonucleotide primers being produced from the KAT enzyme sequences provided herein. See Mullis et al, U.S. Patent 4,683,195; and Mullis, U.S. Patent 4,683,202. The PCR reaction provides a method for selectively increasing the concentration of a particular nucleic acid sequence even when that sequence has not been previously purified and is present only in a single copy in a particular sample. The method can be used to amplify either single- or double-stranded DNA. The essence of the method involves the use of two oligonucleotide probes to serve as primers for the template-dependent, polymerase mediated replication of a desired nucleic acid molecule.

The recombinant DNA molecules of the present invention can be produced through any of a variety of means well known to the expert in the art and

- 9 -

disclosed by, for example, Maniatis et al, *supra*. In order to replicate the KAT enzyme DNA sequences, these must be cloned in an appropriate vector. A vector is a replicable DNA construct. Vectors are used herein either to amplify DNA encoding the KAT enzyme and/or to express DNA which encodes the KAT enzyme. An expression vector is a replicable DNA construct in which a DNA sequence encoding the KAT enzyme is operably linked to suitable control sequences capable of effecting the expression of the KAT enzyme in a suitable host. DNA regions are operably linked when they are functionally related to each other. For example: a promoter is operably linked to a coding sequence if it controls the transcription of the sequence. Amplification vectors do not require expression control domains. All that is needed is the ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants.

DNA sequences encoding KAT enzyme may be recombined with vector DNA in accordance with conventional techniques, including blunt-ended or staggered-ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases. Techniques for such manipulation are disclosed by Maniatis et al, *supra* and are well known in the art.

Expression of the cloned sequence occurs when the expression vector is introduced into an appropriate host cell. If a prokaryotic expression vector is employed, then the appropriate host cell would be any prokaryotic cell capable of expressing the cloned sequences, for example *E. coli*. Similarly, if an

- 10 -

eukaryotic expression vector is employed, then the appropriate host cell would be any eukaryotic cell capable of expressing the cloned sequence. A yeast host may be employed, for example *S. cerevisiae*.  
5 Alternatively, insect cells may be used, in which case a baculovirus vector system may be appropriate. Another alternative host is a mammalian cell line, for example COS-1 cells.

The need for control sequences into the expression vector will vary depending upon the host selected and the transformation method chosen. Generally, control sequences include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA  
10 ribosomal binding sites, and sequences which control the termination of transcription and translation. Vectors useful for practicing the present invention include plasmids, viruses (including phages),  
15 retroviruses, and integrable DNA fragments (i.e., fragments integrable into the host genome by homologous recombination). The vectors replicate and function independently of the host genome, or may, in some instances, integrate into the genome itself.

Expression vectors should contain a promoter which is recognized by the host organism. The promoter sequences of the present invention may be either prokaryotic, eukaryotic or viral. Example of suitable prokaryotic sequences include the  $P_R$  and  $P_L$  promoters of bacteriophage lambda (Hershey, The Bacteriophage Lambda, Ed., Cold Spring Harbor Press,  
25 Cold Spring Harbor, NY (1973); and Hendrix, Lambda II, Ed., Cold Spring Harbor Press, Cold Spring Harbor, NY (1980)); the *trp*, *recA*, heat shock, and *lacZ* promoters of *E. coli* and the SV40 early promoter (Benoit et al, Nature, 290:304-310 (1981)).  
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- 11 -

As far as the Shine-Dalgarno sequence is concerned, preferred examples of suitable regulatory sequences are represented by the Shine-Dalgarno of the replicase gene of the phage MS-2 and of the gene cII of bacteriophage lambda. The Shine-Dalgarno sequence may be directly followed by the DNA encoding KAT and result in the expression of the mature KAT protein.

Alternatively, the DNA encoding KAT may be preceded by a DNA sequence encoding a carrier peptide sequence. In this case, a fusion protein is produced in which the N-terminus of KAT is fused to a carrier peptide, which may help to increase the protein expression levels and intracellular stability, and provide simple means of purification. A preferred carrier peptide includes one or more of the IgG binding domains of Staphylococcus protein A. Fusion proteins comprising IgG binding domains of protein A are easily purified to homogeneity by affinity chromatography, e.g., on IgG-coupled Sepharose. A DNA sequence encoding a recognition site for a proteolytic enzyme such as enterokinase, factor X or procollagenase may immediately precede the sequence for KAT to permit cleavage of the fusion protein to obtain the mature KAT protein.

Moreover, a suitable expression vector includes an appropriate marker which allows the screening of the transformed host cells. The transformation of the selected host is carried out using any one of the various techniques well known to the expert in the art and described in Maniatis et al, *supra*.

One further embodiment of the invention is a prokaryotic host cell transformed with the said expression vector and able to produce, under appropriate culture conditions, the KAT of the invention.

- 12 -

Cultures of cells derived from multicellular organisms are a desirable host for recombinant KAT synthesis. In principal, any eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture, including insect cells. Propagation of such cells in cell culture has become a routine procedure. See Kruse et al, Tissue Culture, Eds., Academic Press (1973). Examples of useful host cell lines are HeLa cells, CHO and COS cell lines. The transcriptional and translational control sequences in expression vectors to be used in transforming vertebrate and invertebrate cells are often provided by viral sources. For example, commonly used promoters are derived from Adenovirus 2, polyoma and SV40. See, e.g. U.S. Patent 4,599,308.

An origin of replication may be provided either by construction of the vector to include an exogenous origin or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter may be sufficient.

Rather than using vectors which contain viral origins of replication, one can transform mammalian cells by the method of cotransformation with a selectable marker and the KAT DNA. An example of a suitable marker is dihydrofolate reductase (DHFR) or thymidine kinase. See U.S. Patent No. 4,399,216.

Cloned genes and vectors of the present invention are useful to transform cells which do not ordinarily express KAT to thereafter express this enzyme. Such cells are useful as intermediates for making recombinant KAT preparations useful for drug screening.

Moreover, genes and vectors of the present invention are useful in gene therapy. For such

- 13 -

purposes, adenovirus vectors as well as retroviral vectors as described in Temin et al, U.S. Patent 4,650,764 and Miller, U.S. Patent 4,861,719 may be employed.

5           Cloned genes of the present invention, and oligonucleotides derived therefrom, are useful for screening for restriction fragment length polymorphism (RFLP) associated with certain disorders.

10           Oligonucleotides of the present invention are useful as diagnostic tools for probing KAT gene expression in various tissues. For example, tissue can be probed in situ with oligonucleotide probes carrying detectable groups by conventional autoradiography techniques to investigate native  
15           expression of this enzyme or pathological conditions relating thereto.

          Genetically modified (transfected) cells have been successfully used for cerebral implantation. Cells transfected with the KAT gene can be useful for  
20           delivering kynurenic acid (or any other KAT product; see below) to the brain. This may prove to be an attractive means to circumvent the blood-brain barrier for kynurenic acid through peripheral administration of kynurenine (or any appropriate substrate of KAT;  
25           see below).

          Transfected cells expressing large quantities of KAT are also useful for the production of neuroactive kynurenic analogs. For example, KAT is capable of forming the potent NMDA receptor antagonist and neuroprotectant 7-chlorokynurenic acid from its  
30           bioprecursor L-4-chlorokynurenine (J. Med. Chem., 37:334-336 (1994)).

          The present invention is explained in greater detail in the following examples. These examples are

- 14 -

intended to be illustrative of the present invention,  
and should not be constructed as limiting thereof.

EXAMPLE 1  
Amino Acid Sequence of Tryptic  
Fragments of the Rat KAT

Protein Purification

Rat KAT was prepared essentially as described by  
Okuno et al, Brain Res., 534:37-44 (1990). The enzyme  
eluted from a Sephacryl S-200 column was separated by  
HPLC on a reverse-phase column (SC18, 250 x 4.6 mm,  
Japan Spectro. Co. Ltd). Elution was performed with  
a gradient of solvent A (70% vol/vol) acetonitrile in  
0.1% trifluoroacetic acid (TFA)) and solvent B  
(0.1% TFA) applied for 40 min at a flow rate of  
1 ml/min.

Trypsin and CNBr Digestion  
and Fragment Purification

500 pmoles of HPLC-purified rat KAT sample were  
digested by trypsin as described (Hugli, In:  
Techniques In Protein Chemistry, Eds., Academic Press,  
Inc., pages 377-391 (1989)) and by CNBr. These  
samples were subjected to reverse-phase HPLC after  
digestion and the resulting peaks collected.

Amino Acid Sequence Analysis

Sequence analysis was performed essentially as  
described (Fabbrini et al, FEBS Lett., 286:91-94  
(1991)). Figure 1 shows the partial amino acid  
sequence of rat KAT: N-terminus of mature KAT, (SEQ  
ID NO:14) a CNBr fragment (SEQ ID NO:15), tryptic  
fragment 112 of KAT (SEQ ID NO:16) and tryptic  
fragment 130 of KAT (SEQ ID NO:17).

EXAMPLE 2

- 15 -

Polymerase Chain Reaction (PCR) CloningRNA extraction

Total RNA from rat kidney was extracted from small quantities of tissue according to the instruction of RNazol™ method (RNazol-Cinna/Biotex Lab., Texas, U.S.A.).

First Strand cDNA Synthesis

First strand cDNA was synthesized from 3mg of total RNA using 2mg oligo polydT (18pb), 4ml of dNTP (2.5mM), 8ml of AMV buffer (TrisHCl pH8.8 250mM/ KCl 200mM/MgCl<sub>2</sub> 50mM/ DTT 20mM) in a final volume of 38.75 ml. The solution was boiled for 3 min at 65°C and throw in ice for 10 min; 0.75 ml of RNasin (40u/ml Promega) and 0.5 ml of AMV Reverse transcriptase (25u/ml Boehringer Mannheim, GmbH, Germany) were added to the cold solution. The reaction was carried on at 42°C for 2h.

Design and Synthesis of Degenerated Oligonucleotides

Since the relative position of tryptic fragments 112 and 130, along the rat KAT primary structure, was unknown four degenerated oligonucleotides 26 bp long were designed and synthesized using a DNA/RNA synthesizer 380B Applied Biosystems. The product of the reaction was purified on Sephadex G50 (Nap 25 Column, Pharmacia).

The sense orientation oligonucleotide, OligoA: (AAYTNTGYCARCARCAYGAYGTNGT) (SEQ ID NO:20) and the anti-sense orientation oligonucleotide, OligoC: (ACNACRTCRTGYTGYTGRCANARRTT) (SEQ ID NO: 21) based on the peptide sequence Asn-Leu-Cys-Gln-Gln-His-Asp-Val-Val (residues 7-15 of fragment 130 (SEQ ID NO:17)) while the sense

- 16 -

orientation oligonucleotide, OligoB:  
(ACNGANARRTTYTGRTCTXATNCCRTC) (SEQ ID NO:22) and the  
corresponding anti-sense oligonucleotide, OligoD:  
(GAYGGNATZGAYCARAAYYTNTCNGT) (SEQ ID NO: 23)  
5 based on the peptide sequence  
Asp-Gly-Ile-Asp-Gln-Asn-Leu-Ser-Val (residues 3-11 of  
fragment 112 (SEQ ID NO:16)) (N=T/C/A/G; Z=T/C/A;  
R=A/G; Y=T/C; X=T/G/A) were synthesized.

#### Polymerase Chain Reaction Condition

10 The first strand cDNA was divided in two  
aliquotes and amplified by PCR as described below.  
The two oligonucleotide, mixture PCR1: oligoA and  
oligoD and PCR2 OligoB and OligoC were used as primer  
in the PCR reaction. 70 ng of template cDNA were  
15 combined with 10 mg of each set of primers, 10 ml of  
10x Taq polymerase buffer (500 mM KCl/100 mM Tris-HCl,  
pH 8.3), 8 ml of 25mM MgCl<sub>2</sub>, 8 ml of a dNTP solution  
(2.5 mM dNTP) and 0.5 ml (2.5 units) of Taq DNA  
polymerase (Perkin Elmer Cetus). The volume was  
20 brought to 100 ml with H<sub>2</sub>O and the mixture was  
overlayed with mineral oil to prevent evaporation.  
The tube was heated to 94°C for 3 min, denaturation  
was carried out for 3 minutes at 94°C, annealing for  
2 min at 60°C and polymerization for 2 min and  
25 30 seconds at 72°C. The cycle was repeated 30 times.

A specific amplification product was observed  
only with PCR1. The product of the amplification was  
a DNA molecule of about 550 bp. The  
PCR1-amplification product was re-amplified  
30 using a new set of oligos, basically with the same  
sequence of oligoA and oligoC with  
SalI linkers and 5'-extra nucleotides.  
OligoE: (GCTAGTCGACACNACRTCRTGYTGYTGRCANARRTT)  
(SEQ ID NO:24) complementary to nucleotides

- 17 -

coding for peptide 130 (SEQ ID NO:17) and OligoF:  
(GATCGTCGACGAYGGNATZGAYCARAAYYTNTCNGT) (SEQ ID NO:25)  
corresponding to nucleotides coding for peptide 112  
(SEQ ID NO:16).

5           After PCR amplification the resulting DNA  
fragment was digested overnight with the restriction  
enzyme Sall and ligated to the Sall site of the  
cloning plasmid pUC 18 (Yanisch-Perron et al, Gene,  
33:103-119 (1985)). The recombinant plasmid was  
10           extracted according to the instruction of the Qiagen  
Plasmid Maxi Protocol; precipitated with PEG and  
denaturated with NaOH 2N.

15           Sequencing was carried out with universal and  
forward primer and subsequently with a series of  
synthetic oligonucleotide primers according to the  
dideoxy chain termination method (Sanger et al, Proc.  
Natl. Acad. Sci. USA, 74:5463-5467 (1977)) using  
Sequenase (United States Biochemicals Corp.,  
Cleveland, OH).

20           Both strands of the insert were sequenced  
revealing an open reading frame of 196 amino acids.  
Part of the two rat KAT peptides that were sequenced  
are coded for the corresponding 588 bp open reading  
frame. This open reading frame is used as probe in  
25           the cDNA library screening described in Example 3.

### EXAMPLE 3 cDNA Library Screening

30           About 500,000 recombinant phages of  $\lambda$ gt11 rat  
kidney cDNA library (Clontec Laboratories, USA) were  
plated on a lawn of *E. coli* Y1090 cells. After an  
overnight growth at 37°C the recombinant phages were  
transferred in duplicate nitrocellulose filters, their  
DNA was denatured, neutralized and baked under vacuum  
at 80°C for 2h. Prehybridization was carried out at

- 18 -

60°C for 4h in 6xSSC (1X SSC:\_\_\_), 5x Denhardt's (1X Denhardt:\_\_\_), 1% SDS, 200 ug/ml salmon sperm DNA. The filters were then hybridized overnight at 60°C in the same mixture with the addition of about  
5 1.5 x 10<sup>6</sup> cpm/ml of labeled probe (see Example 2).

The probe was labeled with (<sup>32</sup>P) dCTP by Multiprime DNA labelling system (Amersham), purified on Nick Column (Pharmacia) and added to the hybridizing solution.

10 The filters were washed at 60°C twice in 2xSSC, 0.1% SDS and ones in 1xSSC, 1%SDS. Filters were exposed to Kodak X-AR film (Eastman Kodak Company, Rochester, N.Y., USA) with intensifying screen at -80°C.

15 Positive phage plaques were isolated and screened again twice in order to isolate single clones.

#### Recombinant Phage DNA Extraction and Sequencing Methods

20 About 50,000 phages of each positive clone were plated on a lawn of *E. coli* Y1090 cells. After an overnight growth at 37°C phages were resuspended in SM buffer (100 mM NaCl/8 mM MgSO<sub>4</sub>/50 mM Tris-HCl, pH 7.5/gelatin 0.001%) and chloroform 0.3%; the suspension was treated with 1 mg of RNase and 1 mg of  
25 DNase. Phage DNA was precipitated with PEG 10%/1M NaCl, extracted with phenol and phenol:chloroform:iso-amyl alcohol and precipitated with PEG again.

30 The phage DNA was digested with EcoRI and the insert was ligated to the EcoRI site of pUC18.

The recombinant plasmid was extracted according to the instruction of Qiagen Plasmid Maxi Protocol; precipitated with PEG and denaturated with 2N NaOH.

- 19 -

Sequencing was carried out with universal and forward primer and subsequently with a series of synthetic oligonucleotide primers according to the dideoxy chain termination method (Sanger et al, supra) using Sequenase (United States Biochemicals Corp., Cleveland, OH).

Three positive clones were isolated, cDNA-1, cDNA-2 and cDNA-3. Both strands of the three cDNAs were sequenced (see Figures 2A-2C, 3A-3D and 4A-4D).

cDNA-1 encodes a deduced protein of 423 amino acid residues, cDNA-2 encodes a deduced protein of 437 amino acid residues and cDNA-3 encodes a deduced protein of 457 amino acid residues.

The three deduced proteins differ only in their N-terminus. Moreover, the cDNA-2 and cDNA-3 clones are not homogeneous, since an alternative 5' sequence introduces an upstream ATG starting codon.

As already said, the longer proteins deduced from the cDNA-2 and cDNA-3 clones present a putative mitochondrial transit peptide in position 1 to 24 (cDNA-2) and in position 1 to 44 (cDNA-3) which is only partially present in the 423 amino acid protein.

#### EXAMPLE 4 Cloning of human KAT

A  $\lambda$  ZapII human brain cDNA library (Stratagene) was screened with a probe representing the N-terminal part of the cDNA-1 encompassing a sequence from amino acid residue 11 to 197 and encoding rat kidney KAT. About 1,350,000 recombinant phages were plated on a lawn of E. coli XL1 blue cells and screening was performed as described in the Example 3.

Positive phage plaques were isolated and screened again twice in order to isolate single clones.

- 20 -

#### Recombinant Phage DNA Extraction and Sequencing Methods

*E. coli* XL1 blue cells were coinfectd with about  $10^5$  phage particles corresponding to the positive clone selected and 1  $\mu$ l of EX Assist helper phage ( $10^6$  pfu/ml). The mixture was incubated at 37°C for 15 min and later incubated with 3 ml of LB for 3 h. Cells were spinned down and the supernatant was heated at 70°C for 15 min. SORL cells at OD600=1 were mixed with the supernatant containing the phagemid pBluescript and incubated for 15 min at 37°C and plated on LB-ampicillin plates (50  $\mu$ g/ml). Single clones were incubated overnight in LB-ampicillin and DNA was extracted according to the instruction of Qiagen Plasmid Maxi Protocol, precipitated with PEG and denaturated with NaOH 2N. Sequencing was carried out with universal and forward primer and subsequently with a series of synthetic oligonucleotide primers according to the dideoxy chain termination method (Sanger et al, *supra*) using Sequenase (United States Biochemicals Corp., Cleveland, OH).

Unfortunately none of the positive clones contained a full length sequence. Therefore, in order to isolate the 5' lacking sequence a RACE protocol was applied.

#### 5' PCR Race

0.5  $\mu$ g of polyA+ RNA from human brain was reverse transcribed with a primer (5'-CAGGGCCTGGAAGGCTGTGA-3') (SEQ ID NO:6) located at the N-terminal part of the longest cDNA clone isolated from the human brain cDNA library. Reaction was carried out as described in Example 2. 20  $\mu$ l were precipitated and resuspended in a mixture containing dATP 0.2 mM, buffer tailing (0.1 M potassium cacodylate pH 6.8, 1 mM CoCl<sub>2</sub>, 100 mM

- 21 -

DTT, 100 µg/ml BSA) and 15 U TdT enzyme (Gibco BRL). After incubation at 37°C for 10 min water was added to a final reaction volume of 250 µl. cDNA was mixed with 25 pmol of oligo (5'-ATAGCCACCAACAGTCACCA-3') (SEQ ID NO:7), 10 p/mol of oligo (5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTTTT-3') (SEQ ID NO:8) and 25 pmol of oligo (5'-GACTCGAGTCGACATCGA-3') (SEQ ID NO:9), 10 µl of 10x Taq polymerase buffer (500 mM KCl/100mM Tris.HCl, pH 8.3), 8 µl of 25 mM MgCl<sub>2</sub>, 8 µl of a dNTP solution (2.5 mM dNTP). The volume was brought to 100 µl with H<sub>2</sub>O. The tube was heated at 95°C for 7 min and 0.5 µl (2.5 U) of Taq polymerase (Perkin Elmer Cetus) were added. Annealing was carried out for 2 min at 58°C and polymerization for 2.5 min at 72°C: The cycle was repeated 40 times. PCR products were blotted on a nitrocellulose filter and hybridized as described in Example 3 with a oligonucleotide probe (5'-ACCACTGACGAAGATCCTGGCAAGTTTCTTTGGGGAGC-3') (SEQ ID NO:10), based on the known sequence of the partial cDNA human clone. Probe was labelled with (γ<sup>32</sup>P) dATP by T4 polynucleotide Kinase (Boehringer) and purified on Nap5 column (Pharmacia). A positive bands (330 bp) and termed 5'-hKAT was re-amplified using oligo with SalI linkers and cloned in pUC18. DNA sequencing was performed on both strands confirming correspondence between the PCR fragment and the lacking 5'-part of the human KAT clone.

## EXAMPLE 5

Expression in Mammalian Cells

The expression plasmid encoding rat KAT was constructed as follows: a) To remove the 5' and the 3' untranslated sequences, as well as the putative mitochondrial targeting peptide, PCR amplification was

- 22 -

performed using two specific oligonucleotides with XhoI linkers. The sense orientation oligonucleotide (5'-TGTCCTCGAGACCATGACCAAACGGCTGCAGGCTCGGA-3') (SEQ ID NO:26) begins at +241 of cDNA-1, whereas the antisense-orientation oligonucleotide (5'-GTACCTCGAGTCAGGGTTGGAGCTCTTTCCACTTG-3') (SEQ ID NO:27) complements the sequence starting from the end of the coding sequence. The XhoI-digested fragment, after being controlled by sequencing, was cloned into the XhoI site of pSVL expression vector (Pharmacia Biotechnology).

The expression plasmid encoding human KAT was constructed as follows. In order to join the two cDNA fragments corresponding to the full length sequence of human KAT two different PCR reactions were carried out: a) 5'hKAT was amplified by PCR using two specific oligonucleotides: a sense primer with XhoI linker (TGTCCTCGAGACCATGGCCAAACAGCTC) (SEQ ID NO:11) and as reverse primer (CAGGGCCTGGAAGGCTGTGA) (SEQ ID NO:6) the oligonucleotide used for the reverse transcription of Race (see Example 4); b) the partial cDNA sequence coding for human KAT obtained after cDNA library screening (Example 4) was PCR amplified using two primers flanking the cloning site: sense primer (GTAATACGACTCACTATAGGGC) (SEQ ID NO:12) and reverse primer (TGTCCTCGAGCGCTCTAGAACTAGTGGATC) (SEQ ID NO:13). The two PCR product were were digested with ApaI, linked together, digested XhoI and cloned into the pSVL vector. COS-1 cells were transfected with 10 ug of pSVL-ratKAT plasmid or pSVL-humankAT by calcium phosphate method (Maniatis et al, *supra*). 72 h after transfection, cells were disrupted by freezing and thawing and after centrifugation the supernatant was used for KAT, glutamine transaminase K and cysteine conjugate  $\beta$ -lyase activities.

- 23 -

EXAMPLE 6  
Kynurenine Amino Transferase, Glutamine  
Amino Transferase K and Cysteine Conjugate  
 $\beta$ -lyase Activities

Kynurenine transaminase assay

The reaction mixture (100  $\mu$ l) contained 70  $\mu$ M pyridoxal phosphate, 5 mM pyruvate, 3mM kynurenine, and KAT sample in 0.17 M potassium phosphate buffer, pH 8.1, and was incubated at 37°C for 30 min and 1 h. Reaction was stopped by adding 20  $\mu$ l TCA 50% and the precipitate was removed by centrifugation. The supernatant was analyzed by HPLC with a C18 column (Vydac 201TP54, 25x4.6 cmxmm) at 1 ml/min, equilibrated with 5 mM acetic acid, 5% methanol, 0.1% heptane sulfonic acid, pH 3.0, and kynurenic acid was eluted with 50mM acetic acid, 5% methanol, 0.5% heptane sulfonic acid, pH 4.5. Absorbance at 243 nm was measured.

Glutamine Transaminase K Assay

Glutamine transaminase K activity was measured as described by Cooper and Meister (Methods Enzymol., 113:344-349 (1985)).

Cysteine Conjugate  $\beta$ -lyase Assay

The  $\beta$ -lyase assay was a coupled assay as described by Abraham and Cooper (1991). The product of the  $\beta$ -lyase reaction (pyruvate) was assayed by measuring the oxidation of NADH during the transformation of pyruvate to lactate catalyzed by alanine dehydrogenase. The reaction mixture (200  $\mu$ l in a microtiter plate) contained 2 mM S-(1,2-dichlorovinyl)-L-cysteine (DCVC), 0.5 mM MTB, 0.1 mM PLP and the enzyme in 100 mM Tris buffer pH 8.8, and was incubated at 37°C for 5, 10, 15 min prior the addition of 0.3 mM NADH, 7.3 U/ml alanine

- 24 -

dehydrogenase, and ammonium acetate 0.1 M. Absorbance at 340 nm was measured using a microplate reader (Cervus uv900) and NADH concentration was calculated using a  $\epsilon = 4200 \text{ M}^{-1}$ .

## EXAMPLE 7

Amino Acid Sequence of Tryptic  
Fragments of the Human KAT

Human KAT was prepared essentially as described by Baran et al, J. Neurochem., 62:730-738 (1994).

500 pmoles of the purified human KAT sample were digested by trypsin as described (Hugli, In: Techniques in Protein Chem., pages 377-391, Eds., Academic Press, Inc., (1989)). Briefly, human KAT was desalted using SMART system equipped with Fast desalting column equilibrated in 10 mM ammonium bicarbonate. After the chromatography step sample was concentrated to a final volume of % ml. Cysteine residues in the molecule were reduced in 8 M urea, 10 mM DTT at 50°C for 15 min then the alkylation was carried out with 20 mM iodoacetic acid for 15 min at room temperature. After this time the sample solution was diluted to have a final urea concentration of 2 M and the sample was digested overnight with trypsin (Boehringer) with an enzyme: substrate ratio 1:25. Peptides coming from digestion were analyzed by RP-HPLC using a Vydac C18 column and a linear gradient from 5 to 65% eluent B during 60 min, where eluent A was 0.1% trifluoroacetic acid (TFA) in water and eluent B was 0.07% TFA, 95% acetonitrile. Eluted peaks were manually collected, concentrated using a vacuum speedvac (Savant) and then loaded onto a 477 N-terminal protein sequencer (ABI, Perkin Elmer) for protein sequence determination.

- 25 -

Sequence analysis was performed essentially as described (Fabbrini et al, FEBS Lett., *supra*). Figure 6 shows the amino acid sequence of three peptides of human KAT, namely F11 (SEQ ID NO:2),  
5 F13 (SEQ ID NO:3) and F14 (SEQ ID NO:4).

While the invention has been described in detail, and with reference to specific embodiments thereof, it will be apparent to one of ordinary skill in the art that various changes and modifications can be made  
10 therein without departing from the spirit and scope thereof.

- 26 -

IN THE CLAIMS:

Claim 1. An isolated DNA sequence encoding a KAT enzyme selected from the group consisting of:

(a) an isolated DNA sequence which encodes  
5 rat KAT enzyme;

(b) an isolated DNA sequence which hybridizes to isolated DNA sequences of (a) above and which encodes a mammalian KAT enzyme; and

(c) an isolated DNA sequence differing from  
10 the isolated DNA sequences of (a) and (b) above in codon sequence due to the degeneracy of the genetic code, and which encodes a KAT enzyme.

Claim 2. An isolated DNA sequence according to Claim 1 which encodes rat KAT enzyme.

15 Claim 3. An isolated DNA sequence according to Claim 1 which encodes rat KAT enzyme, which further comprises the sequence of the clone cDNA-1 (SEQ ID NO:18).

20 Claim 4. An isolated DNA sequence according to Claim 1 which encodes rat KAT enzyme, which further comprises the sequence of the clone cDNA-2 (SEQ ID NO:19).

25 Claim 5. An isolated DNA sequence according to Claim 1 which encodes rat KAT enzyme, which further comprises the sequence of the clone cDNA-3 (SEQ ID NO:5).

Claim 6. A vector comprising a cloned DNA sequence as defined in any one of Claims 1 to 5.

30 Claim 7. A vector comprising a cloned DNA sequence as defined in any one of Claims 1 to 5, wherein the vector is a plasmid.

Claim 8. A vector comprising a cloned DNA sequence as defined in any one of Claims 1 to 5, wherein the vector is a virus.

- 27 -

Claim 9. A vector comprising a cloned DNA sequence as defined in any one of Claims 1 to 5, wherein the vector is a retrovirus.

5 Claim 10. A host cell transformed with a vector comprising a cloned DNA sequence as defined in any one of Claims 1 to 5.

10 Claim 11. A host cell transformed with a vector comprising a cloned DNA sequence as defined in any one of Claims 1 to 5, wherein the cell is a mammalian cell.

Claim 12. An oligonucleotide probe capable of selectively hybridizing to a DNA sequence comprising a portion of a gene coding for a KAT enzyme.

15 Claim 13. An oligonucleotide probe capable of selectively hybridizing to a DNA sequence comprising a portion of a gene coding for a KAT enzyme, wherein the probe is capable of serving as a polymerase chain reaction extension primer.

20 Claim 14. An oligonucleotide probe capable of selectively hybridizing to a DNA sequence comprising a portion of a gene coding for a KAT enzyme, wherein the probe is labelled with a detectable group.

25 Claim 15. An oligonucleotide probe capable of selectively hybridizing to a DNA sequence comprising a portion of a gene coding for a KAT enzyme, wherein the probe is labelled with a detectable group, wherein the detectable group is a radioactive atom.

30 Claim 16. An isolated and purified KAT enzyme which is coded for by a DNA sequence according to any one of Claims 1 to 5.

Claim 17. A method of screening drugs comprising transforming cells useful for drug screening with a vector comprising a cloned DNA sequence as defined in any one of Claims 1-5.

- 28 -

Claim 18. A method of screening drugs comprising using the host cell according to any one of Claims 10 or 11 to screen drugs.

5 Claim 19. A method of gene therapy comprising cloning a vector useful for gene therapy, which comprises a cloned DNA sequence as defined in any one of Claims 1 to 5.

10 Claim 20. A method of gene therapy comprising using the host cell according to any one of Claims 10 to 11 for gene therapy.

Claim 21. A method of gene therapy comprising using the host cell according to any one of Claims 10 to 11 for cerebral implantation.

15 Claim 22. A method of gene therapy comprising using the host cell according to any one of Claims 10 to 11 for delivering kynurenic acid to the brain.

20 Claim 23. A method of gene therapy comprising cloning an adenovirus vector useful for gene therapy, which comprises a cloned DNA sequence as defined in any one of Claims 1 to 5.

25 Claim 24. A method of gene therapy comprising isolating a DNA sequence as defined in any one of Claims 1 to 5, wherein said DNA sequence is useful for gene therapy.

30 Claim 25. A method of screening for restriction fragment length polymorphism, comprising cloning a DNA sequence as defined in any one of Claims 1 to 5, wherein said clone is useful for RFLP screening.

Claim 26. A method of screening for RFLP, comprising using an oligonucleotide probe according to any one of Claims 12 to 15 for RFLP screening.

Claim 27. A method of probing for KAT gene expression, comprising using an oligonucleotide probe

- 29 -

according to any one of Claims 12 to 15 to probe for KAT gene expression.

5           Claim 28. A method of producing neuroactive kynurenic analogs comprising transforming cells useful for producing neuroactive kynurenic analogs with a vector comprising a cloned DNA sequence as defined in any one of Claims 1 to 5.

10           Claim 29. A method of producing neuroactive kynurenic analogs comprising using the host cell according to any one of Claims 10 to 11 to produce neuroactive kynurenic analogs.

1/17

N - terminal

Leu-Gln-Ala-X-X-Leu-Asp-Gly-Ile-Asp-Gln-Asn-

Leu-X-Val-Glu-Phe-Gly-Lys-Thr-X-Glu-Tyr

CNBr fragment

X-X-Leu-Pro-Gly-Ala-Glu-Asp-Gly-Pro-Tyr-Asp-Arg

-Arg-X-Ala

Tryptic fragment 112

Arg-Leu-Asp-Gly-Ile-Asp-Gln-Asn-Leu-Ser-Val-

Glu-Phe-Gly

Tryptic fragment 130

X-Glu-Leu-Glu-Leu-Val-Ala-Asn-Leu-Cys-Gln-Gln-His

Asp-Val-Cys-Ile-Ser-Asp-Glu-Val-Tyr-Gln-Gln-Val-Tyr-

Asp-Leu-Gly-His-Gln

**FIG. 1**

2/17

10	20	30	40	50	60	
12345678901234567890123456789012345678901234567890						
AAACTGACCAAGGAGTATGATCAATCCCGTCCAGCCTCCGAGCCTGCAGCCGGTTTGGTCA						60
TGGTGAGCTGCTTCAGCTAACAATTGCACTGACAGTGTCTCTTGAGCCAAGTTGCTTCTGG						120
GOGGAAGTAGTCCATCTAGGGCTCGGCGCTCTTTAAAGAAACAGACTTCTGCAACCTTGGG						180
ACTACGTTTGGGGTGGCGGCTATTGGAAGGAGCAGGCAATTGTTAGCTGAAGCAGCTC						240
ACCATGACCAAACGGCTGCAGGCTCGGAGGCTGGACGGGATTGATCAAAACCTCTGGGTG						300
MetThrLysArgLeuGlnAlaArgArgLeuAspGlyIleAspGlnAsnLeuTrpVal						
GAGTTTGGCAAACCTGACCAAGGAGTATGAAGTGGTGAAGTTGGGTGAGGGCTTCCTGAC						360
GluPheGlyLysLeuThrLysGluTyrAspValValAsnLeuGlyGlnGlyPheProAsp						
TTCTCGCCTCCGGACTTTGCAAAGCAAGCTTTTCAGCAGGCTACCAGTGGGAAGTTTCATG						420
PheSerProProAspPheAlaThrGlnAlaPheGlnGlnAlaThrSerGlyAsnPheMet						
CTCAACCAGTACACCAGGGCATTTGGTTACCCAACACTGACAAACGTCCTGGCAAGTTTC						480
LeuAsnGlnTyrThrArgAlaPheGlyTyrProProLeuThrAsnValLeuAlaSerPhe						
TTTGGCAAGCTGCTGGGACAGGAGATGGACCCACTCACGAATGTGCTGGTGACAGTGGGT						540
PheGlyLysLeuLeuGlyGlnGluMetAspProLeuThrAsnValLeuValThrValGly						
GCCTATGGGGCCTTGTTTCACAGCTTTTCAGGCGCTGGTGGATGAAGGAGATGAGGTCATC						600
AlaTyrGlyAlaLeuPheThrAlaPheGlnAlaLeuValAspGluGlyAspGluValIle						
ATCATGGAAACCTGCTTTTGAAGTGTATGAACCCATGACAATGATGGCTGGAGGTTGCCCT						660
IleMetGluProAlaPheAspCysTyrGluProMetThrMetMetAlaGlyGlyCysPro						
GTGTTGCTGACTCTGAAGCCGAGCCCTGCTCCTAAGGGGAAACTGGGAGCCAGCAATGAT						720
ValPheValThrLeuLysProSerProAlaProLysGlyLysLeuGlyAlaSerAsnAsp						
TGGCAACTGGATCCTGCAGAACTGGCCAGCAAGTTTACACCTCGCACCAAGTTCTGGTTC						780
TrpGlnLeuAspProAlaGluLeuAlaSerLysPheThrProArgThrLysValLeuVal						
CTCAACACACCCCAACAACCTTTAGGAAAGGTATTCTCTAGGATGGAGCTGGAGCTGGTG						840
LeuAsnThrProAsnAsnProLeuGlyLysValPheSerArgMetGluLeuGluLeuVal						
GCTAATCTGTGCCAGCAGCAAGATGTGCTGTGTCATCTCTGATGAGGTTCTACCAGTGGCTG						900
AlaAsnLeuCysGlnGlnHisAspValValCysIleSerAspGluValTyrGlnTrpLeu						

FIG. 2A

SUBSTITUTE SHEET (RULE 26)

3/17

10	20	30	40	50	60	
12345678901234567890123456789012345678901234567890						
GTCTATGACGGGCAOCAGCACGTCAGCATOGCCAGCCTCCCTGGCATGTGGGATOGGAOC						960
ValTyrAspGlyHisGlnHisValSerIleAlaSerLeuProGlyMetTrpAspArgThr						
CTGACCATCGGCAGTGCAGGCAAAAGCTTCAGTGGCACTGGCTGGAAGGTGGGCTGGGTC						1020
LeuThrIleGlySerAlaGlyLysSerPheSerAlaThrGlyTrpLysValGlyTrpVal						
ATGGGTCCAGATAACATCATGAAGCAOCTGAGGACAGTGCACCAGAATTCTATCTTCCAC						1080
MetGlyProAspAsnIleMetLysHisLeuArgThrValHisGlnAsnSerIlePheHis						
TGCCCCACCCAGGCCCCAGGCTGCAGTAGCCAGTGCCTTTGAGCGGGAGCAGCAACACTTT						1140
CysProThrGlnAlaGlnAlaAlaValAlaGlnCysPheGluArgGluGlnGlnHisPhe						
GGACAAOCCAGCAGCTACTTTTTGCAGCTGCCACAGGCCATGGAGCTGAACCGAGACCAC						1200
GlyGlnProSerSerTyrPheLeuGlnLeuProGlnAlaMetGluLeuAsnArgAspHis						
ATGATCCGTAGCCTGCAGTCAGTGGGCTCAAGCTCTGGATCTCCAGGGGAGCTACTTC						1260
MetIleArgSerLeuGlnSerValGlyLeuLysLeuTrpIleSerGlnGlySerTyrPhe						
CTCATTCGACACATCTCAGACTTCAAGAGCAAGATGCOCTGACCTGCCCCGAGCTGAGGAT						1320
LeuIleAlaAspIleSerAspPheLysSerLysMetProAspLeuProGlyAlaGluAsp						
GAGCCTTATGACAGACGCTTTTGCCAAGTGGATGATCAAAAACATGGGCTTGGTGGGCATC						1380
GluProTyrAspArgArgPheAlaLysTrpMetIleLysAsnMetGlyLeuValGlyIle						
OCTGTCTCCACATTCTTCAGTGGCCCCATCAGAAGGACTTTGAOCCACTACATCCGATTTC						1440
ProValSerThrPhePheSerArgProHisGlnLysAspPheAspHisTyrIleArgPhe						
TGTTTTGTCAAGGACAAGGCCACACTCCAGGCCATGGATGAGAGACTGCGCAAGTGGAAA						1500
CysPheValLysAspLysAlaThrLeuGlnAlaMetAspGluArgLeuArgLysTrpLys						
GAGCTCCAACCTTGAGGAGGCTGCCCTCAGCCCCACCTCGAACACAGGCTCAGCTATGC						1560
GluLeuGlnPro						
CTTAGCACAGGGATGGCACTGGAGGGGCCAGCTGTGTGACTGCGCATGTTTCCAGAAAAG						1620
AGGCCATGTCTTGGGGTTGAAGCCATCCTTTCCAGTGTCCATCTGGACTATTGGGTTG						1680
GGGGCCAGTTCTGGGTCTCAGCCTACTCCTCTGTAGGTTGCCTGTAGGGTTTGTATTGTT						1740
TCTGGCCTCTCTGCCTGGGGCAGGAAAGGGTGGGAATATCAGGCCCGGTACCACCTTAGCC						1800

FIG. 2B

4/17

10	20	30	40	50	60	
<u>123456789012345678901234567890123456789012345678901234567890</u>						
CTGOCGAGGCTCTGTGGCTTCCTACATCCTCTCCTGTGACCTCAGGATGTTGCTACTGT						1860
TCCTAATAAAGTTTTAAGTTATTAGGAOCTCA						1893

FIG. 2C

5/17

10	20	30	40	50	60	
123456789012345678901234567890123456789012345678901234567890						
GGGOGACTCTAGATTTTTTTTTTTTTTTTACCTTCTACCTTTTATGTCAAGTGAACCATG						60
GTOCTACAGGCTGCTGACAAGCTTGGCTGAGCAGGGATCCCAGGGGOGTGGCAGGAGAT						120
GAGGAAGGGTTGCTGGGAGGGCTTGGGCTCTTCCTTGAGAAGACAGCAAATGTATOCAGC						180
CTAGATTAAAGGGTAGGGCATCCCCATCCCTGTCACTGGGCTAGATCTCAGAGCCCCAC						240
ATTAAAGACTGCTAATGGGTCAGAAATGGGGGTCCCTTAGATGGGGGTAGCCAGCAAGGC						300
OCTCCCTCCAGTGTTCTCATTCTGTTCGGTTTCATTTGTTGTGTCCAGGGACGGTGAAG						360
CAGATACCAGTCTCAAGCCCCAGGGTGCAGGAAGACGGGAAATGGGAAAATGGAAACATT						420
CTCAAGTGACCAGAGCACTCTGCCGGGGACAAAAGACTTTGCCTTGAACGGGTAGTGGA						480
GAAGCTACAAACCCCAGGTCCCAGTGGGCTGATTGACTTAGGGTCTCAGCTGGCCCCAAA						540
CTCAGTGTGTAGATCAGACTGATCTCAAACCTCACAGAGATCTCCCTGCTTTGCTGCTG						600
AGTOCTGGGATTAAAGGCATGAATCACAGTACCTGGTGCCTTTTCTTTAAAAAGCTCACC						660
MetAsnHisSerThrTrpCysLeuPhePheLysLysLeuThr						

FIG. 3A

6/17

10	20	30	40	50	60	
12345678901234567890123456789012345678901234567890						
ATGACCAAACGGCTGCAGGCTGGAGGCTGGACGGGATTGATCAAAAOCTCTGGGTGGAG						720
MetThrLysArgLeuGlnAlaArgArgLeuAspGlyIleAspGlnAsnLeuTrpValGlu						
MetThrLysArgLeuGlnAlaArgArgLeuAspGlyIleAspGlnAsnLeuTrpValGlu						
TTTGGCAAACCTGACCAAGGAGTATGACGTGCTGAACCTTGGGTCAGGCTTCCCTGACTTC						780
PheGlyLysLeuThrLysGluTyrAspValValAsnLeuGlyGlnGlyPheProAspPhe						
PheGlyLysLeuThrLysGluTyrAspValValAsnLeuGlyGlnGlyPheProAspPhe						
TCGCTCCGGACTTTTGCAACGCAAGCTTTTCAGCAGGCTACCAAGTGGGAACCTTCATGCTC						840
SerProProAspPheAlaThrGlnAlaPheGlnGlnAlaThrSerGlyAsnPheMetLeu						
SerProProAspPheAlaThrGlnAlaPheGlnGlnAlaThrSerGlyAsnPheMetLeu						
AACAGTACACCAAGGCAATTTGGTTAOCACCACTGACAAACGTCTGCAAGTTTCTTT						900
AsnGlnTyrThrArgAlaPheGlyTyrProProLeuThrAsnValLeuAlaSerPhePhe						
AsnGlnTyrThrArgAlaPheGlyTyrProProLeuThrAsnValLeuAlaSerPhePhe						
GGCAAGCTGCTGGGACAGGAGATGGACCCACTCAOGAATGTGCTGGTGACAGTGGGTGCC						960
GlyLysLeuLeuGlyGlnGluMetAspProLeuThrAsnValLeuValThrValGlyAla						
GlyLysLeuLeuGlyGlnGluMetAspProLeuThrAsnValLeuValThrValGlyAla						
TATGGGGCCTTGTTTCAAGCTTTTCAGGCOCTGGTGGATGAAGGAGATGAGGTCATCATC						1020
TyrGlyAlaLeuPheThrAlaPheGlnAlaLeuValAspGluGlyAspGluValIleIle						
TyrGlyAlaLeuPheThrAlaPheGlnAlaLeuValAspGluGlyAspGluValIleIle						
ATGGAACCTGCTTTTGACTGTTATGAACCCATGACAATGATGGCTGGAGGTTGCCCTGTG						1080
MetGluProAlaPheAspCysTyrGluProMetThrMetMetAlaGlyGlyCysProVal						
MetGluProAlaPheAspCysTyrGluProMetThrMetMetAlaGlyGlyCysProVal						
TTCGTGACTCTGAAGCCGAGCCCTGCTCTAAGGGGAACTGGGAGCCAGCAATGATTGG						1140
PheValThrLeuLysProSerProAlaProLysGlyLysLeuGlyAlaSerAsnAspTrp						
PheValThrLeuLysProSerProAlaProLysGlyLysLeuGlyAlaSerAsnAspTrp						
CAACTGGATCCTGCAGAACTGGCCAGCAAGTTCACAOCTCGCACCAAGCTCTGGTCCTC						1200
GlnLeuAspProAlaGluLeuAlaSerLysPheThrProArgThrLysValLeuValLeu						
GlnLeuAspProAlaGluLeuAlaSerLysPheThrProArgThrLysValLeuValLeu						
AACACACCCAACAACCCCTTTAGGAAAGGTATCTCTAGGATGGAGCTGGAGCTGGTGGCT						1260
AsnThrProAsnAsnProLeuGlyLysValPheSerArgMetGluLeuGluLeuValAla						
AsnThrProAsnAsnProLeuGlyLysValPheSerArgMetGluLeuGluLeuValAla						
AATCTGTGCCAGCAGCAGCATGTGCTGTGCATCTCTGATGAGGTCTACCAAGTGGCTGGTC						1320
AsnLeuCysGlnGlnHisAspValValCysIleSerAspGluValTyrGlnTrpLeuVal						
AsnLeuCysGlnGlnHisAspValValCysIleSerAspGluValTyrGlnTrpLeuVal						

FIG. 3B

SUBSTITUTE SHEET (RULE 26)

7/17

10	20	30	40	50	60	
123456789012345678901234567890123456789012345678901234567890						
TATGACGGGCACCAGCACGTCAGCATCGCCAGCCTCCCTGGCATGTGGGATCGGACCCCTG						1380
TyrAspGlyHisGlnHisValSerIleAlaSerLeuProGlyMetTrpAspArgThrLeu						
TyrAspGlyHisGlnHisValSerIleAlaSerLeuProGlyMetTrpAspArgThrLeu						
AACATCGGCAGTGCAGGCAAAAGCTTCAGTGCCTGGCTGGAAGGTGGGCTGGGTCATG						1440
ThrIleGlySerAlaGlyLysSerPheSerAlaThrGlyTrpLysValGlyTrpValMet						
ThrIleGlySerAlaGlyLysSerPheSerAlaThrGlyTrpLysValGlyTrpValMet						
GGTCAGATAACATCATGAAGCACTGAGGACAGTGCACCAAGATTCCTATCTTCCACTGC						1500
GlyProAspAsnIleMetLysHisLeuArgThrValHisGlnAsnSerIlePheHisCys						
GlyProAspAsnIleMetLysHisLeuArgThrValHisGlnAsnSerIlePheHisCys						
CCACCCAGGCCAGGCTGCAGTAGCCAGTGCCTTTGAGCGGGAGCAGCAACACTTTGGA						1560
ProThrGlnAlaGlnAlaAlaValAlaGlnCysPheGluArgGluGlnGlnHisPheGly						
ProThrGlnAlaGlnAlaAlaValAlaGlnCysPheGluArgGluGlnGlnHisPheGly						
CAOCCAGCAGCTACTTTTTCAGCTGOCACAGGCCATGGAGCTGAACCGAGACCACATG						1620
GlnProSerSerTyrPheLeuGlnLeuProGlnAlaMetGluLeuAsnArgAspHisMet						
GlnProSerSerTyrPheLeuGlnLeuProGlnAlaMetGluLeuAsnArgAspHisMet						
ATCOGTAGCCTGCAGTCAGTGGGCTCAAGCTCTGGATCTCCAGGGGAGCTACTTCTCTC						1680
IleArgSerLeuGlnSerValGlyLeuLysLeuTrpIleSerGlnGlySerTyrPheLeu						
IleArgSerLeuGlnSerValGlyLeuLysLeuTrpIleSerGlnGlySerTyrPheLeu						
ATTGCAGACATCTCAGACTTCAAGAGCAAGATGCCTGACCTGCCCGGAGCTGAGGATGAG						1740
IleAlaAspIleSerAspPheLysSerLysMetProAspLeuProGlyAlaGluAspGlu						
IleAlaAspIleSerAspPheLysSerLysMetProAspLeuProGlyAlaGluAspGlu						
CCTTATGACAGACGCTTTGCCAAGTGGATGATCAAAAACATGGGCTTGGTGGGCATCCCT						1800
ProTyrAspArgArgPheAlaLysTrpMetIleLysAsnMetGlyLeuValGlyIlePro						
ProTyrAspArgArgPheAlaLysTrpMetIleLysAsnMetGlyLeuValGlyIlePro						
GTCTCCACATTCTTCAGTGGGCCCCATCAGAAGGACTTTGACCACTACATCCGATTCTGT						1860
ValSerThrPhePheSerArgProHisGlnLysAspPheAspHisTyrIleArgPheCys						
ValSerThrPhePheSerArgProHisGlnLysAspPheAspHisTyrIleArgPheCys						
TTTGTCAAGGACAAGGCCACACTCCAGGCCATGGATGAGAGACTGCGCAAGTGGAAAGAG						1920
PheValLysAspLysAlaThrLeuGlnAlaMetAspGluArgLeuArgLysTrpLysGlu						
PheValLysAspLysAlaThrLeuGlnAlaMetAspGluArgLeuArgLysTrpLysGlu						
CTCCAACCTGAGGAGGCTGCCCTCAGCCCCACCTCGAACACAGGCTCAGCTATGCCTT						1980
LeuGlnPro						
LeuGlnPro						

FIG. 3C

SUBSTITUTE SHEET (RULE 26)

8/17

10	20	30	40	50	60	
123456789012345678901234567890123456789012345678901234567890						
AGCACAGGGGATGGCACTGGAGGGGCCAGCTGTGTGACTGCGCATGTTTCAGAAAAGAGG						2040
CCATGTCTTGGGGGTGAAGCCATCCTTTCCAGTGTCCATCTGGACTATTGGGTTGGGG						2100
GCCAGTTCTGGGTCTCAGCCTACTCCTCTGTAGGTGCGCTGTAGGGTTTTGATTGTTTCT						2160
GGCCTCTCTGCTGGGGCAGGAAAGGGTGGAATATCAGGCGCGGTACACCTTAGCCCTG						2220
CCGAGGCTCTGTGGCTTCTCTACATCTTCTCCTGTGACCTCAGGATGTTGCTACTGTTCC						2280
TAATAAAGTTTTAAGTTATTAGGA						2304

FIG. 3D

9/17

10	20	30	40	50	60	
123456789012345678901234567890123456789012345678901234567890						
AAACTGACCAAGGAGTATGATCAATCCCGTCCAGCCTCCGAGCCTGCAGCCGTTTGGTCA						60
TGGTGAGCTGCTTCAGCTAACAATTGCACTGACAGTGCTCTTGAGCCAAGTTGCTTCTGG						120
GCGGAAGTAGTCCATCTAGGGCTCGGCTCTTTAAAGAAACAGACTTCTGCAACCTTGGG						180
ACTACGTTTGGGGTCGOCGGCTATTGGAACGGAGCAGCGCAATTGTTAGCTGAAGCAGAAC						240
TGTGTGTGGACTCAGGOCCTGGCTTGGAGCCATTTTCTGGGCTAGGCTGTCTGCCCCCTCT						300
GTCCCTCTGGAGGGGAAGCCTGCAGTGCCTGTGGACCTACCTCAGAGGCATGTTTACGCAG						360
					MetPheArgSe	
TGCAGCAGCCCTCTCGGTGCACCTGATGTGGCCACTCTGGGGAAGGAAAGCTGGAGCCTC						420
rAlaAlaAlaLeuSerValHisLeuMetTrpProLeuTrpGlyArgLysAlaGlyAlaSe						
ACTCACCGGTGGCTTGCACCAAGTCTCTACCATGACCAAACGGCTGCAGGCTCGGAGCCT						480
rLeuThrArgCysLeuHisGlnSerLeuThrMetThrLysArgLeuGlnAlaArgArgLe						
					MetThrLysArgLeuGlnAlaArgArgLe	
GGACGGGATTGATCAAAACCTCTGGGTGGAGTTTGGCAAACCTGACCAAGGAGTATGACGT						540
uAspGlyIleAspGlnAsnLeuTrpValGluPheGlyLysLeuThrLysGluTyrAspVa						
uAspGlyIleAspGlnAsnLeuTrpValGluPheGlyLysLeuThrLysGluTyrAspVa						
CGTGAACCTTGGGTACGGGCTTCCTGACTTCTCGCCTCCGGAAGTTTGCAACGCAAGCTTT						600
lValAsnLeuGlyGlnGlyPheProAspPheSerProProAspPheAlaThrGlnAlaPh						
lValAsnLeuGlyGlnGlyPheProAspPheSerProProAspPheAlaThrGlnAlaPh						
TCAGCAGGCTACCAAGTGGGAACCTTCATGCTCAACCAAGTACACCAAGGCATTGGTTACCC						660
eGlnGlnAlaThrSerGlyAsnPheMetLeuAsnGlnTyrThrArgAlaPheGlyTyrPr						
eGlnGlnAlaThrSerGlyAsnPheMetLeuAsnGlnTyrThrArgAlaPheGlyTyrPr						

**FIG. 4A**

SUBSTITUTE SHEET (RULE 26)

10/17

10	20	30	40	50	60	
123456789012345678901234567890123456789012345678901234567890						
ACC	ACTGACAA	AGTTC	TTTGCCA	AGCTGCT	GGGACAGG	ATGGACCC 720
oProLeu	ThrAsnVal	LeuAlaSer	PhePheGly	LysLeuLeu	GlyGlnGlu	MetAspPr
oProLeu	ThrAsnVal	LeuAlaSer	PhePheGly	LysLeuLeu	GlyGlnGlu	MetAspPr
ACTC	ACGAATGT	GCTGGT	GACAGT	GGGTGC	CTATGGGG	COCTTG 780
oLeuThr	AsnValLeu	ValThrVal	GlyAlaTyr	GlyAlaLeu	PheThrAla	PheGlnAl
oLeuThr	AsnValLeu	ValThrVal	GlyAlaTyr	GlyAlaLeu	PheThrAla	PheGlnAl
CCTG	TGGATGA	AGGAGAT	GAGGTC	ATCATCAT	GGAACCTG	CTTTGACT 840
aLeuVal	AspGluGly	AspGluVal	IleIleMet	GluProAla	PheAspCys	TyrGluPr
aLeuVal	AspGluGly	AspGluVal	IleIleMet	GluProAla	PheAspCys	TyrGluPr
CATG	ACAATGAT	GGCTGG	AGGTTG	CCCTGIGT	TCGTGACT	CTCTGAAG 900
oMetThr	MetMetAla	GlyGlyCys	ProValPhe	ValThrLeu	LysProSer	ProAlaPr
oMetThr	MetMetAla	GlyGlyCys	ProValPhe	ValThrLeu	LysProSer	ProAlaPr
TAAG	GGAAGT	GGGAGCC	AGCAATG	ATTGGCA	ACTGGATC	CTGCAGAA 960
oLysGly	LysLeuGly	AlaSerAsn	AspTrpGln	LeuAspPro	AlaGluLeu	AlaSerLy
oLysGly	LysLeuGly	AlaSerAsn	AspTrpGln	LeuAspPro	AlaGluLeu	AlaSerLy
GTTCA	CACTCG	CAACCA	AGGTCCT	TGGTCTCA	CACACAC	CCCAACA 1020
sPheThr	ProArgThr	LysValLeu	ValLeuAsn	ThrProAsn	AsnProLeu	GlyLysVa
sPheThr	ProArgThr	LysValLeu	ValLeuAsn	ThrProAsn	AsnProLeu	GlyLysVa
ATTCT	CTAGGAT	GGAGCT	GGAGCT	GGTGGCTA	ATCTGTG	CCAGCAG 1080
lPheSer	ArgMetGlu	LeuGluLeu	ValAlaAsn	LeuCysGln	GlnHisAsp	ValValCy
lPheSer	ArgMetGlu	LeuGluLeu	ValAlaAsn	LeuCysGln	GlnHisAsp	ValValCy
CATCT	CTGATG	AGGCTA	CCAGTGG	CTGGTCTA	TGACGGGC	ACCAGCAC 1140
sIleSer	AspGluVal	TyrGlnTrp	LeuValTyr	AspGlyHis	GlnHisVal	SerIleAl
sIleSer	AspGluVal	TyrGlnTrp	LeuValTyr	AspGlyHis	GlnHisVal	SerIleAl
CAGCT	CCCTGG	CATGTG	GGATGG	GACCTG	ACCATGG	GCAGTGC 1200
aSerLeu	ProGlyMet	TrpAspArg	ThrLeuThr	IleGlySer	AlaGlyLys	SerPheSe
aSerLeu	ProGlyMet	TrpAspArg	ThrLeuThr	IleGlySer	AlaGlyLys	SerPheSe
TGCC	ACTGGCT	GGAAGGT	GGGCTGG	GGTCATG	GGTCCAG	ATAACAT 1260
rAlaThr	GlyTrpLys	ValGlyTrp	ValMetGly	ProAspAsn	IleMetLys	HisLeuAr
rAlaThr	GlyTrpLys	ValGlyTrp	ValMetGly	ProAspAsn	IleMetLys	HisLeuAr
GACAG	TGCACC	AGAATT	CTATCTT	CCACTG	CCCCAC	CCAGGCC 1320
gThrVal	HisGlnAsn	SerIlePhe	HisCysPro	ThrGlnAla	GlnAlaAla	ValAlaGl
gThrVal	HisGlnAsn	SerIlePhe	HisCysPro	ThrGlnAla	GlnAlaAla	ValAlaGl

FIG. 4B

SUBSTITUTE SHEET (RULE 26)

11/17

10	20	30	40	50	60	
123456789012345678901234567890123456789012345678901234567890						
GTGCTTTGAGCGGGAGCAGCAACACTTTGGACAACCCAGCAGCTACTTTTTGCAGCTGCC						1380
nCysPheGluArgGluGlnGlnHisPheGlyGlnProSerSerTyrPheLeuGlnLeuPr						
nCysPheGluArgGluGlnGlnHisPheGlyGlnProSerSerTyrPheLeuGlnLeuPr						
ACAGGCCATGGAGCTGAACCGAGACCACATGATCCGTAGCCTGCAGTCAGTGGGGCTCAA						1440
oGlnAlaMetGluLeuAsnArgAspHisMetIleArgSerLeuGlnSerValGlyLeuLy						
oGlnAlaMetGluLeuAsnArgAspHisMetIleArgSerLeuGlnSerValGlyLeuLy						
GCTCTGGATCTCCAGGGGAGCTACTTCCTCATTTGCAGACATCTCAGACTTCAAGAGCAA						1500
sLeuTrpIleSerGlnGlySerTyrPheLeuIleAlaAspIleSerAspPheLysSerLy						
sLeuTrpIleSerGlnGlySerTyrPheLeuIleAlaAspIleSerAspPheLysSerLy						
GATGCCTGACCTGCCCCGAGCTGAGGATGAGCCTTATGACAGACGCTTTGCCAAGTGGAT						1560
sMetProAspLeuProGlyAlaGluAspGluProTyrAspArgArgPheAlaLysTrpMe						
sMetProAspLeuProGlyAlaGluAspGluProTyrAspArgArgPheAlaLysTrpMe						
GATCAAAAACATGGGCTTGGTGGGCATCCCTGCTCCACATTCTTCAGTCGGCCCCATCA						1620
tIleLysAsnMetGlyLeuValGlyIleProValSerThrPhePheSerArgProHisGly						
tIleLysAsnMetGlyLeuValGlyIleProValSerThrPhePheSerArgProHisGly						
GAAGGACTTTGACCACTACATCCGATTCTGTTTTGTCAAGGACAAGGCCACACTCCAGGC						1680
nLysAspPheAspHisTyrIleArgPheCysPheValLysAspLysAlaThrLeuGlnAl						
nLysAspPheAspHisTyrIleArgPheCysPheValLysAspLysAlaThrLeuGlnAl						
CATGGATGAGAGACTGCGCAAGTGGAAAGAGCTCCAACCTGAGGAGGCTGCCCTCAGCC						1740
aMetAspGluArgLeuArgLysTrpLysGluLeuGlnPro						
aMetAspGluArgLeuArgLysTrpLysGluLeuGlnPro						
CCAACCTOGAACACAGGCTCAGCTATGCCTTAGCACAGGGATGGCACTGGAGGGCCCCAGC						1800
TGTGTGACTGCGCATGTTTCCAGAAAAGAGGCCATGCTCTGGGGGTTGAAGCCATCCTTT						1860
CCCAGTGTCCATCTGGACTATTTGGGTTGGGGGCCAGTTCTGGGTCTCAGCCTACTCCTCT						1920
GTAGGTTGCCTGTAGGGTTTTGATTGTTTCTGCCCTCTCTGCCTGGGGCAGGAAAGGGTG						1980

FIG. 4C

12/17

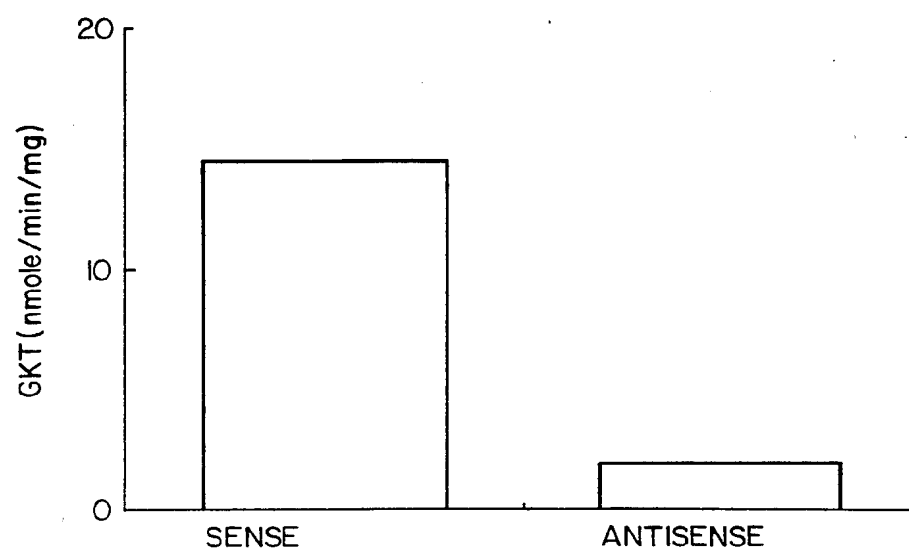
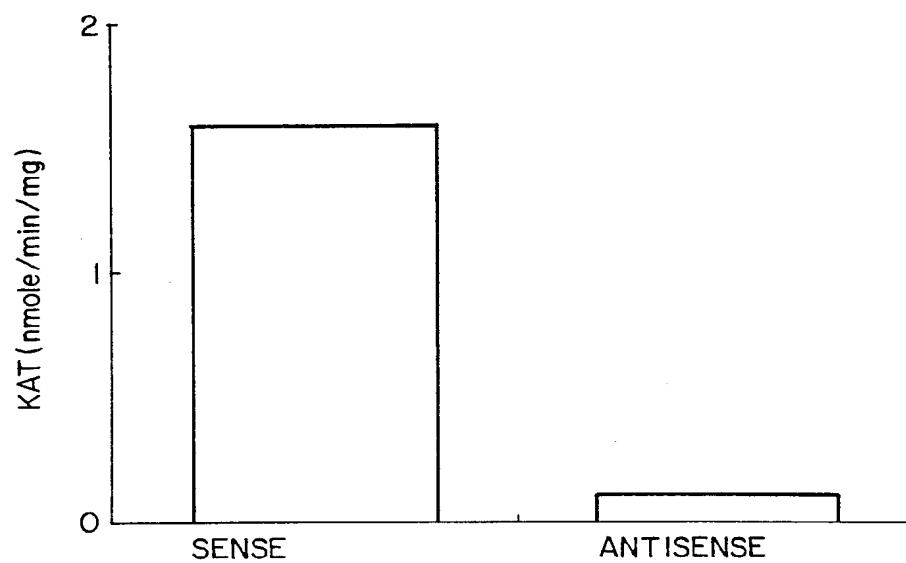
10	20	30	40	50	60	
123456789012345678901234567890123456789012345678901234567890						
GAATATCAGGCCCCGGTACCACTTAGCCCTGCOGAGGCTCTGTGGCTTCTCTACATCTTC						2040

TCCTGTGACCTCAGGATGTTGCTACTGTTCTAATAAAAGTTTTAAGTTATTAGGACCCCTC 2100

C 2101

**FIG. 4D**

13/17

**FIG. 5A****FIG. 5B**

14/17

Tryptic fragment F11

Thr-Phe-Ser-Ala-Thr-Gly-XXX-Lys

The sequence corresponds to positions 191-198 of the protein deduced from the human brain cDNA sequence

Tryptic fragment F13

Ala-Leu-Val-Leu-Asn-Thr-Pro-Asn-Asn-Pro-Leu-Gly-Lys

The sequence corresponds to positions 120-132 of the protein deduced from the human brain cDNA sequence

Tryptic fragment F14

Glu-Gln-Leu-Leu-Phe-Arg

The sequence corresponds to positions 238-243 of the protein deduced from the human brain cDNA sequence

**FIG. 6**

15/17

10	20	30	40	50	60	
12345678901234567890123456789012345678901234567890						
CTTAATGTTTTTTAGAGCTCACCATGGCCAAACAGCTGCAGGCCCCAAGGCTAGACGGGAT						60
		MetAlaLysGlnLeuGlnAlaArgArgLeuAspGlyIl				
CGACTACAACCCCTGGGTGGAGTTTGTGAACTGGCCAGTGAGCATGACGTCGTGAACTT						120
eAspTyrAsnProTrpValGluPheValLysLeuAlaSerGluHisAspValValAsnLe						
GGGCCAGGGCTTCCCGGATTTCCACCACCAGACTTTGCCGTGGAAGCCTTTCAGCACGC						180
uGlyGlnGlyPheProAspPheProProProAspPheAlaValGluAlaPheGlnHisAl						
TGTCAGTGGAGACTTCATGCTTAACCAGTACACCAAGACATTTGGTTACCCACCACTGAC						240
aValSerGlyAspPheMetLeuAsnGlnTyrThrLysThrPheGlyTyrProProLeuTh						
GAAGATCCTGGCAAGTTTCTTTGGGGAGCTGCTGGGTGAGGAGATAGACCCGCTCAGGAA						300
rLysIleLeuAlaSerPhePheGlyGluLeuLeuGlyGlnGluIleAspProLeuArgAs						
TGTGCTGGTGACTGTTGGTGGCTATGGGGCCCTGTTACAGCCTTCCAGGCCCTGGTGGAA						360
nValLeuValThrValGlyGlyTyrGlyAlaLeuPheThrAlaPheGlnAlaLeuValAs						
CGAAGGAGACGAGGTCATCATCATCGAACCCTTTTTTGACTGCTACGAGCCCATGACAAT						420
pGluGlyAspGluValIleIleIleGluProPhePheAspCysTyrGluProMetThrMe						
GATGGCAGGGGGTTCGTCCTGTGTTTGTGTCCCTGAAGCCGGGTCCCATCCAGAATGGAGA						480
tMetAlaGlyGlyArgProValPheValSerLeuLysProGlyProIleGlnAsnGlyGl						
ACTGGGTTCCAGCAGCAACTGGCAGCTGGACCCCATGGAGCTGGCCGGCAAATTCACATC						540
uLeuGlySerSerSerAsnTrpGlnLeuAspProMetGluLeuAlaGlyLysPheThrSe						
ACGCACCAAAGCCCTGGTCCTCAACACCCCCAACAACCCCTGGGCAAGGTGTTCTCCAG						600
rArgThrLysAlaLeuValLeuAsnThrProAsnAsnProLeuGlyLysValPheSerAr						
GGAAGAGCTGGAGCTGGTGGCCAGCCTTTGCCAGCAGCATGACGTGGTGTGTATCACTGA						660
gGluGluLeuGluLeuValAlaSerLeuCysGlnGlnHisAspValValCysIleThrAs						

FIG. 7A

SUBSTITUTE SHEET (RULE 26)

16/17

10	20	30	40	50	60	
12345678901234567890123456789012345678901234567890						
TGAAGTCTACCAGTGGATGGTCTACGACGGGCACCAGCACATCAGCATTGCCAGCCTCCC						720
pGluValTyrGlnTrpMetValTyrAspGlyHisGlnHisIleSerIleAlaSerLeuPr						
TGGCATGTGGGAACGGACCCTGACCATCGGCAGCGCCGGCAAGACCTTCAGCGCCACTGG						780
oGlyMetTrpGluArgThrLeuThrIleGlySerAlaGlyLysThrPheSerAlaThrGl						
CTGGAAGGTGGGCTGGGTCTGGGTCCAGATCACATCATGAAGCACCTGCGGACCGTGCA						840
yTrpLysValGlyTrpValLeuGlyProAspHisIleMetLysHisLeuArgThrValHi						
CCAGAACTCCGTCTTCCACTGCCCCACGCAGAGCCAGGCTGCAGTAGCCGAGAGCTTTGA						900
sGlnAsnSerValPheHisCysProThrGlnSerGlnAlaAlaValAlaGluSerPheGl						
ACGGGAGCAGCTGCTCTTCCGCCAACCCAGCAGCTACTTTGTGCAGTTCCCGCAGGCCAT						960
uArgGluGlnLeuLeuPheArgGlnProSerSerTyrPheValGlnPheProGlnAlaMe						
GCAGCGCTGCCGTGACCACATGATACGTAGCCTACAGTCAGTGGGCCTGAAGCCCATCAT						1020
tGlnArgCysArgAspHisMetIleArgSerLeuGlnSerValGlyLeuLysProIleIl						
CCCTCAGGGCAGCTACTTCCTCATCACAGACATCTCAGACTTCAAGAGGAAGATGCCTGA						1080
eProGlnGlySerTyrPheLeuIleThrAspIleSerAspPheLysArgLysMetProAs						
CTTGCTGGAGCTGTGGATGAGCCCTATGACAGACGCTTCGTCAAGTGGATGATCAAGAA						1140
pLeuProGlyAlaValAspGluProTyrAspArgArgPheValLysTrpMetIleLysAs						
CAAGGGCTTGGTGGCCATCCCTGTCTCCATCTTCTATAGTGTGCCACATCAGAAGCACTT						1200
nLysGlyLeuValAlaIleProValSerIlePheTyrSerValProHisGlnLysHisPh						
TGACCACTATATCCGCTTCTGTTTTGTGAAGGATGAAGCCACGCTCCAGGCCATGGACGA						1260
eAspHisTyrIleArgPheCysPheValLysAspGluAlaThrLeuGlnAlaMetAspGl						
GAAGCTGCGGAAGTGGAAGGTGGAAGTCTAGCCCTGAAGTCACGCCTTGGCCCTGACATC						1320
uLysLeuArgLysTrpLysValGluLeu...						

FIG. 7B

17/17

10	20	30	40	50	60	
123456789012345678901234567890123456789012345678901234567890						
CCCACATGCCCCGAGAGATCCTCTTTGAGTGTCTGTCTTTGTCCAGGTTTCAGACATTTC						1380
TAGGTTGGGGAAGATGCTATTGGGAAACCTCTTCTCCGTGACACAGAATGTTCTGGGTGG						1440
GAGCCGCCCTTCTTCATCTTAGAGAACCAAGTACCTCCTGTCTGAAAGGTGAGGGTGGCC						1500
TGACCTGGGCCTCTCCCTGCCCCCTCCATAGGTGGGTTTGTAGGGTCTTGTGTTGCTTCTG						1560
GTCTCTCCAGGCTTGGCTGAGACGGACGGTAGACTTCCACCATGTACCGATCACATCCCA						1620
ACTCTGCATGGCCCCCTGCTAAGGCTCAGGTATAACCTCACCTTCCCTGGCTCATCTTGGC						1680
CTTGGGGAGTTGCCTTTAGGCTTGAGTCCTCAAGCCTCTCCTTTTCGTCCATAATAAAAT						1740
GGGAATTC						1748

FIG. 7C

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/07855

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 9/10, 9/88, 15/54, 15/60, 15/70; C12Q 1/68; A61K 31/70  
US CL : 435/6, 193, 232, 252.3, 320.1; 536/23.2; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 193, 232, 252.3, 320.1; 536/23.2; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG

search terms: KAT, kynurenine aminotransferase, rat

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- A	JOURNAL OF BIOCHEM, Vol. 279, issued 1991, MAWAL ET AL., "Purification and properties of kynurenine aminotransferase from rat kidney", PAGES 595-599, SEE ENTIRE DOCUMENT.	16 --- 1-15, 17-29
X --- A	BIOCHIMICA ET BIOPHYSICA ACTA, Vol. 743, issued 1983, TAKEUCHI ET AL, "Purification, characterization and identification of rat liver mitochondrial kynurenine aminotransferase with alpha-aminoadipate aminotransferase", PAGES 323-330, SEE ENTIRE DOCUMENT	16 --- 1-15, 17-29

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

10 OCTOBER 1995

Date of mailing of the international search report

01 NOV 1995

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

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Telephone No. (703) 308-0196

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/07855

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/07855

### BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-11, 17-18 and 28-29, drawn to DNA sequences and first method of use in screening assay.

Group II, claims 12-15, drawn to oligonucleotide probes.

Group III, claim 16, drawn to a KAT enzyme.

Group IV, claims 19-24, drawn to a method of gene therapy.

Group V, claims 25-27, drawn to a method of screening for RFLP's.

The inventions listed as Groups I, and IV or V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I comprises DNA and its first method of use. The subsequent groups IV and V comprise separate methods of use of the DNA compound. These methods of use are unrelated to each other and to the first method of use in their technique and/or additional components, and thus do not relate to a single inventive concept.

The inventions listed as Groups III, and I-II or IV-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The enzyme of Group III is chemically and physically separate from the DNA of Groups I and II, and thus also the methods of using this in Groups IV-V. Thus, the inventions of Groups I-II and IV-V lack a corresponding special technical feature with the enzyme of Group III.

The inventions listed as Groups II, and I or IV-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group II is directed to oligonucleotide probes, while Group I is directed to whole DNA encoding the enzyme, which has a different activity and use from the oligonucleotides. Thus, Groups IV-V lack the same corresponding special technical feature as well as Group I, and do not relate to Group II.